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Genetic, Biochemical and Reproductive Toxicity of Cat's Claw after Chronic Treatment in Swiss Albino Mice

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Abstract: It was reported that Cat's claw possess beneficial and adverse effects, which are ascribed to alkaloids, terpenes and steroids. However, there is a paucity of reports on toxicity of Cat's claw. Although, Cat's claw has folkloric significance for its beneficial uses, there is dearth of literature on reproductive toxicity. In view of the traditional significance of Cat's claw, its immense use and a paucity of literature, it was found meaningful to explore its effect on (1) fertility and dominant lethal mutations (2) morphology of spermatozoa (3) cytogenetic analysis of testicular chromosomes (4) biochemical analysis of proteins, nucleic acids, malondialdehyde (MDA) and nonprotein sulfhydryl groups (NP-SH) in the testes and (5) pituitary-gonadal hormones in plasma. Themethodology included oral treatment of mice with different doses (58, 116 and 233 mg/kg/day) of Cat's claw for 90 days. The treatment caused reduction in fertility of female mice mated to treated male mice, induced pre-implantation loss, caused changes in sperm abnormality, reduced the MDA levels of testis, the levels of human-chorionic gonadotropin increased and testosterone levels were decreased, however there was no effect on testicular chromosomes and dominant lethal mutations. The adverse effects on parameters of study in reproduction might be attributed to the impact onmechanistic parameters including biochemistry of nucleic acids,malondialdehyde,nonprotein sulfhydryl groups and hormones observed in the present study. The observed effects appears to be relatedto the oxidant and antioxidant activity of alkaloids, terpenes and steroids present in Cat's claw.

Key words: Cat's Claw · Dominant Lethal Mutations · Pre-Implantation Loss · Nucleic Acids · Malondialdehyde · Nonprotein Sulfhydryl Groups · Hormones

INTRODUCTION

Cat's claw is the most popular Peruvian plant used in folk medicine against treatment of cancer, arthritis, digestive complaints, inflammatory and immune system disorders, Crohn's disease, colitis, rheumatoid arthritis and osteoarthritis [1-3]. While both the species of Cat's claw (Uncaria tomentosa and Uncaria guianensis) provide effective antioxidant and anti-inflammatory activities, the latter is more potent [4, 5]. Aqueous extract of

Uncaria tomentosa is shown to have beneficial effects on DNA repair and immune function [6]. Experiments on anticancer activity revealed Cat's claw to possess cancer chemopreventive activity, as shown by the inhibition of Epstein-Barr virus-early antigen (EBV-EA) in Raji cells exposed to the tumor promoter phorbol ester [7]. In human breast cancer cell line, it is found to show antiproliferative activity [4] and inhibition of cell division and NF-kappa B activity without inducing cell death [8]. The plant has been useful in reducing the secondary effects of radiation and chemotherapy in cancer victims as well [1]. Rizzi *et al.* [9] found that, ingestion of U. Tomentosa decoction by smokers decreased the mutagenicity induced in S. typhimurium TA98 and TA100 in the urine of the subjects. Phytopreparations with immunostimulatory properties are known to increase the frequency of sister chromatid exchanges in cultured peripheral blood mononuclear cells

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(PBMC), an effect associated with a slight increase of [3H] thymidine uptake in the DNA of isolated lymphocytes [10].

The chemical constituents reported from Cat's claw are alkaloids (glucoindole alkaloids, isopteropodine, rynchophyiline, pteropodine and isopteropodine, pentacyclicoxidoles and tetracyclic oxidoles [1, 11, 12] triterpene glycosides, quinovic acid glycosides [13, 14], steroids like beta-sitosterol, stigmasterol and campesterol [15]. In view of immense use of Cat's claw against a wide range of health problems and a paucity of literature on genetic and biochemical effects on reproductive toxicity, the present study aimed to analyse the genetic, biochemical and reproductive toxicity of Cat's claw on sex cells in mice.

MATERIALS AND METHODS

Cat's claw was used as the test herbal product in the present study. It is Nature's Way Products Inc., Springville, Utah 84663, USA. The product is marketed by GNC (General Nutrition Corporation, USA) in Saudi Arabia. It is available in the form of capsules. Each capsule contains 175 mg of Cat's claw dried extract (Bark) standardized to 4% alkaloids and 160 mg Cat's claw (Bark). The other contents are millet and gelatin. The suggested dose is one capsule, two times daily for human use.

Dose Determination: The doses selected for different studies on Cats' claw were based on the MTD (3.73 g/kg., body weight) value (evaluated in our laboratory) and preliminary experiments [16]. The different doses selected for cats' claw were 58, 116 and 233 mg/kg., body weight/day, corresponding to 1/64, 1/32 and 1/16, respectively of the MTD value. The duration of treatment was 90 days (sub-chronic). The dosage form was aqueous suspension and the route of administration was gastric intubation (oral) in all the experiments.

Experimental Groups: Male and female mice (Swiss albino, bred at Experimental Animal Care Center, College of Pharmacy, King Saud University, Saudi Arabia) aged 6-8 weeks, weighing 25-30 g were used. The animals were maintained under standard conditions of temperature (23 ± 1° C), light/dark cycle (12 h/12 h) and relative humidity.

The experimental groups of mice consisted of: group 1, control (tap water); group 2, Cats' claw (58 mg/kg/day); group 3, Cats' claw (116 mg/kg/day), group 4, Cat's claw (233 mg/kg/day). The different parameters of study were (i) fertility index in male mice (ii) evaluation of dominant lethal assay, (iii) analysis of spermatozoa abnormalities; (iv) study on chromosomal aberrations; (v) biochemical evaluation (vi) estimation of hormones.

Estimation of Fertility Index and Dominant Lethal Assay: The methods described in male anti-fertility study [17] and dominant lethal assay [18, 19] was followed to evaluate (i) the rate of fertility in male mice (ii) induction of pregnancy (iii) total and pre-implantation loss and (iv) embryo-toxicity. After the treatment, each male mouse in the treated and control groups was caged with three females, which were allowed to stay with the male for one week. The female mice were necropsied 13 days following the mid-week of their caging and presumptive mating and the number of pregnant mice was recorded to determine percent fertility[20]. The pre-implantation loss was calculated by comparing the number of implantations per pregnant female in the treated and control groups. The dead implants per pregnant female were determined to obtain the post-implantation embryonic loss [19].

Evaluation of Spermatozoa Abnormalities: The spermatozoa were obtained by making small cuts in caudae epididymis and vas deferens placed in 1 ml of modified Krebs Ringer-bicarbonate buffer (pH 7.4). To evaluate the spermatozoa abnormalities, the sperm suspension was stained with eosin; smears were made on slides, air-dried and made permanent. Coded slides were examined by bright-field microscope with an oil immersion lens. The different spermatozoa abnormalities screened were amorphous, banana shaped, swollen acrosome, triangular head, macrocephaly and rotated head [19-22].

Cytogenetic Analysis of Germ Cells: The mice were sacrificed after the last day of the treatment [19, 20]. The testes were removed in an isotonic sodium citrate solution and the seminiferous tubules were teased to form a cell suspension. The suspension was centrifuged and the pellet re-suspended in the hypotonic citrate solution. After the second centrifugation the supernatant was discarded and the pellet suspended in a fixative (Methanol and acetic acid, 3:1). The chromosomal preparations were made by the air drying technique [19, 23]. The coded slides were stained in Giemsa solution and screened for the aberrations including aneuploids, autosomal univalents, sex-univalents and polyploids.

Biochemical Evaluation: The frozen samples of testes were used for estimation of proteins, ribose nucleic acid (RNA) and deoxyribose nucleic acid (DNA), MDA and NP-SH levels.

Estimation of Total Proteins and Nucleic Acids: Total proteins were estimated by the modified Lowry method of Schacterle and Pollack [24]. Bovine serum albumin was used as standard. The method described by Bregman[25] was used to determine the levels of nucleic acids. Testes were homogenized and the homogenate was suspended in ice-cold trichloroacetic acid (TCA). After centrifugation, the pellet was extracted with ethanol. The levels of DNA were determined by treating the nucleic acid extract with diphenylamine reagent and reading the intensity of blue color at 600 nm. For quantification of RNA, the nucleic acid extract was treated with orcinol and the green color was read at 660 nm. Standard curves were used to determine the amounts of nucleic acids present.

Determination of MDA Concentrations: The method described by Ohkawa *et al.* [26] was used. Testes were homogenized in TCA solution and the homogenate suspended in thiobarbituric acid. After centrifugation the optical density of the clear pink supernatant was read at 532 nm. Malondialdehydebis (Dimethyl acetal) was used as an external standard.

Quantification of the NP-SH Levels: The method of Sedlak and Lindsay [27] was used to determine the levels of NP-SH. The testes were homogenized in ice cold 0.02 M ethylene-o-amine tetra acetic acid disodium (EDTA) before mixing with TCA. The homogenate was centrifuged at 3000g. The supernatant was suspended in tris buffer, 5-5'-dithiobis-(2 nitrobenzoic acid) (DTNB) and read at 412 nm against reagent blank with no homogenate.

Estimation of Hormones: The plasma samples were analyzed to estimate the concentrations of human chorionic gonadotropin, luteinizing hormone, follicle stimulating hormone, estradiol, prolactin and testosterone. The analysis was done by direct immune-enzymatic colorimetric method based on ELISA. The procedure used for each hormone was according to the method described for the particular kit [28].

Statistical Analysis: The different studies undertaken were statistically analyzed by Analysis of Variance. Some Parameters on reproductive performance were analyzed using a Chi-square test.

RESULTS

Effect of Cat's Claw on Fertility Index in Male Mice: The sub-chronic treatment with Cat's claw showed 50% reduction in the fertility of female mice mated to treated male mice at the high dose (Table 1).

Effect of Cat's Claw on the Induction of Dominant Lethal Assay: In mating week 1, the sub-chronic treatment of male mice with Cat's claw significantly ($P<0.05$) decreased the percent pregnant female mice at a dose of 116 mg/kg., body weight/day). There were no significant changes in the mean implants/pregnant female, except decrease of total and live implants at the lower dose (58 mg/kg., body weight/day) of Cat's claw. The mean implants/pregnant female (total and live) were increased to the normal levels at the higher doses. There was an increase in the dead implants/pregnant female mice and percent dead embryos at the high dose of Cat/s claw. These changes were statistically insignificant ($P>0.05$) (Table 2).

In mating week 2, the sub-chronic treatment of male mice with Cat's claw caused no significant changes in percent pregnant female mice, total and live implants per pregnant female mice. However, the dead implants per pregnant female mice and percent dead embryos were found to increase at the high dose. These changes were statistically insignificant (Table 2).

Effect of Cat's Claw on Epididymal Spermatozoal Morphology: The sub-chronic treatment with Cat's claw at the high dose caused a statistically significant increase in the frequency of rotated head-shaped abnormality of the spermatozoa ($P<0.05$) and an increase in the total spermatozoa abnormality percent in the epididymis ($P<0.05$). An increasing trend of abnormalities was also observed in the frequency of spermatozoa types, amorphous, banana-shaped, triangular head and macrocephali (Table 3).

Effect of Cat's Claw on Testicular Chromosomes: The sub-chronic treatment with Cat's claw induced a trend increase in the frequency of different chromosomal aberrations such as, aneuploids, autosomal univalents, sex-univalents and the total chromosomal abnormalities, but these results were statistically insignificant (Table 4).

Effect of Cat's claw on Proteins, RNA, DNA, MDA and NP-SH Concentrations: The testicular concentrations of protein, RNA and DNA were not affected at any of the doses of Cat's claw. The depletion of NP-SH in the testis

Table 1: Effect of Cat's claw on fertility index in male and female mice after sub-chronic treatment in male Swiss albino mice

	Fertility index in male mice			Fertility index in female mice		
Treatment and dose (mg/kg. Body weight/ day)	No. of male mice mated	No. of fertile male mice	Fertility percent	No. of female mice mated	No. of fertile female mice	Fertility percent
Control (0.3 ml tap water/mouse)	10	10	100	10	9	90
Cat's claw (58.3)	10	10	100	10	8	80
Cat's claw (116.6)	10	10	100	10	7	70
Cat's claw (233)	10	9	90	10	5	50

10 mice (male or female) were used in each group

P>0.05 (Chi square test)

Table 2: Effect of Cat's claw on the induction of dominant lethal mutations after sub-chronic treatment in male Swiss albino mice

	Mating week 1					Mating week 2				
		Mean Implants/pregnant female ± S.E.					Mean Implants/pregnant female ± S.E.			
Treatment and dose (mg/kg. Body weight/ day)	Pregnant females	Total	Live	Dead	Percent dead embryos	Pregnant females	Total	Live	Dead	Percent dead embryos
Control (0.3 ml tap water/mouse)	30/30(100.0)	11.17±0.43	10.57±0.47	0.60±0.16	18/335(5.37)	27/30(90.0)	9.47±0.85	9.03±0.82	0.43±0.12	13/284(4.57)
Cat's claw (58)	25/30(83.33)	9.50±0.87	8.80±0.83	0.70±0.23	21/285(7.37)	23/30(76.67)	9.20±1.03	8.77±0.99	0.43±0.10	13/276(4.71)
Cat's claw (116)	24/30*(80.0)	10.77±1.13	10.27±1.08	0.53±0.12	16/323(4.95)	26/30(86.67)	10.70±0.85	10.40±0.85	0.30±0.09	9/321(2.80)
Cat's claw (233)	27/30(90.0)	11.47±0.86	10.43±0.82	1.03±0.24	31/344(9.01)	26/30(86.67)	10.20±0.84	9.50±0.80	0.70±0.19	21/306(6.86)

Figures between parentheses denote percent.

A total of 10 male and 30 females were used in each group

P>0.05 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test)

*P<0.05 (Chi square test)

Table 3: Effect of Cat's claw on sperm motility, count and abnormality in Swiss albino mice after sub-chronic treatment

Different Spermatozoal abnormalities screened/Total	Treatments and dose (mg/kg. Body weight/day)/percent sperm motility, sperm count and percent sperm abnormalities (Mean ± S.E.)			
	Control (tap water, 0.3 ml/mouse/day)	Cat's claw (58.30)	Cat's claw (116.60)	Cat's claw (233)
Amorphous	0.42±0.06	0.41±0.07	0.49±0.05	0.57±0.04
Banana shaped	0.29±0.03	0.34±0.06	0.41±0.08	0.43±0.05
Swollen achrosome	0.42±0.05	0.38±0.05	0.45±0.06	0.44±0.04
Triangular head	0.41±0.11	0.36±0.05	0.39±0.03	0.67±0.06
Macrocephali	0.27±0.04	0.22±0.03	0.29±0.03	0.39±0.06
Rotated head	0.12±0.02	0.08±0.02	0.12±0.02	0.22±0.02*
Total abnormalities	1.93±0.19	1.78±0.17	2.15±0.17	2.76±0.08*
Total sperms screened	5176	5013	5105	5100

Five mice were used in each group

*P<0.05; **P<0.01 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters)

Table 4: Effect of Cat's claw on testis chromosomes in Swiss albino mice after sub-chronic treatment

Different chromosomal abnormalities screened/Total	Treatments and dose (mg/kg. Body weight/day)/percent percent chromosomal aberrations (Mean ± S.E.)			
	Control (tap water, 0.3 ml/mouse/day)	Cat's claw (58)	Cat's claw (116)	Cat's claw (233)
Aneuploids	2.61±0.52	2.90±0.61	3.79±0.57	4.36±1.06
Autosomal univalents	2.98±0.53	3.76±0.80	4.09±0.41	4.39±0.41
Sex-univalents	2.96±0.68	4.34±1.11	3.40±0.55	4.09±0.64
Polyploids	3.23±0.79	3.02±0.44	2.68±0.29	3.28±0.47
Translocations	--	--	--	--
Total-percent aberrations	11.71±1.20	14.03±2.20	13.96±0.57	16.66±0.74
Total stages screened	500	550	525	500

Five mice were used in each group

P>0.05 (One-way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for different parameters).

Table 5: Effect of Cat's claw on Proteins, RNA, DNA, MDA and NP-SHconcentrations in testicular cells of Swiss albino mice after Sub-chronic treatment

	Testicular tissue (Mean±S.E.)				
Treatment Dose (mg/kg. Body weight)	Proteins (mg/100 mg tissue)	RNA (µg/100mg tissue)	DNA (µg/100mg tissue)	MDA (nmol/g wet tissue)	NP-SH (nmol/100 mg wet tissue)
Control (tap water, 0.3 ml/mouse)	14.14±0.54	509.51±17.28	222.62±10.42	202.93±7.81	135.36±9.38
Cat's claw (58)	13.88±0.77	484.57±13.89	211.90±11.65	169.25±8.28*	128.97±5.21
Cat's claw (116)	14.58±0.44	495.61±23.04	187.75±9.40	161.81±11.34*	133.26±7.52
Cat's claw (233)	13.89±0.69	476.66±23.78	201.09±16.48	170.04±6.20**	127.99±3.97

Five mice were used in each group

*P<0.05; **P<0.01 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done individually for male, female and different parameters)

Table 6: Effect of Cat's claw on certain pituiary-gonadal hormones in plasma of male and female Swiss albino mice after sub-chronic treatment

Determination of pituitary-gonadal hormones in plasma	Treatments and dose (mg/kg Body weight/day)/Values of hormones in plasma (Mean ± S.E)			
	Control (tap water, 0.3 ml/mouse/day)	Cat's claw (58)	Cat's claw (116)	Cat's claw (233)
Human-Chorionic Gonadotropin	1.07±0.33	2.43±0.22**	5.62±0.32***	6.32±0.52***
Leutenizing hormone	1.40±0.62	1.30±0.16	2.06±0.81	1.49±0.09
Follicle-Stimulating Hormone	1.20±0.43	1.26±0.42	1.28±0.53	2.16±0.11
Estradiol	0.24±0.09	0.29±0.06	0.30±0.06	0.40±0.89
Prolactin	2.20±1.00	1.53±0.85	1.85±0.94	2.53±0.92
Testosterone	5.00±0.58	2.32±0.92*	1.31±0.52**	2.53±0.80*

Five mice were used in each group

*P < 0.05; **P<0.01: ***P<0.001 (One way ANOVA and Post hoc Tukey-Kramer multiple comparison test was done)

was also not significant. However, the treatment was found to significantly decrease the MDA levels in the testis (P<0.05) at low and medium doses and (P<0.01) at the high dose (Table 5).

Effect of Cat's Claw on Certain Pituitary-Gonadal Hormones: The plasma levels of human chorionic gonadotropin were increased significantly (P<0.01) at low and (P<0.001) at medium and high doses of Cat's claw after sub-chronic treatment in male mice. The treatment was also found to decrease the levels of testosterone at low and high (P<0.05) and medium (P<0.01) doses (Table 6)

DISCUSSION

The results obtained on the fertility index in male mice after sub-chronic treatment showed its influence on fertility in female mice which was reduced to 50%, 70% and 80% corresponding to high, medium and low doses, respectively. However, these results are statistically insignificant. Nogueira Neto *et al.* [29] found Cat's claw to possess contraceptive effect in rats with experimental endometriosis. Added to this, the observation of depleted testosterone levels in the tests in the present study might have also played a role in the reduction of fertility.

The results on the dominant lethal assay revealed decrease of percent pregnant female mice in both first and second weeks of mating; the mean live and total implants were increased at the high dose in the second week of mating. However the dead implants per pregnant female mice and the percent dead embryos were found to potentially increase at this dose. It is interesting to note that the mean total and live implants were found to decrease at the lower dose and these changes were replenished at the higher doses. The percent dead implants and the mean dead implant per pregnant female mice were found to increase by the treatment. The tendency of an increase of pre-implantation loss at the lower dose and the subsequent replenishment at the higher doses is the property explained in the decreased oxidant potentials observed in the male gonads after sub-chronic treatment with Cat's claw. Nogueira Neto *et al.* [29] showed reduction of corpus luteum by Cat's claw. This might have interfered with the production of progesterone required for implantation. Furthermore, the results obtained after sub-chronic treatment showed a significant increase in the frequency of rotated head-shaped abnormality and the total spermatozoa abnormality. The results on abnormal sperms might be related to the depletion of testosterone in the plasma after sub-chronic treatment.These changes might also be

related to the observed pre-implantation loss. The treatment with Cat's claw failed to induce significant changes in the chromosomal aberrations after sub-chronic treatment. There is a paucity of literature on the effect of Cat's claw on chromosomes. However, our results cytogenetic analysis of chromosomes confirms other reports on the results on mutagenicity. The extracts and fractions of Cat's claw bark showed no mutagenic effect in different strains of Salmonella typhimurium with and without metabolic activation [9].The authors also found that the decoction of U. Tomentosa ingested daily for 15 days by a smoker decreased the mutagenicity induced in S. typhimurium TA98 and TA100 by the subject's urine [9]. Valerio and Gonzales [30] also found lack of evidence to demonstrate genotoxic potential and mutagenic activity in rodents. Nevertheless, alkaloid-rich preparations from Uncaria tomentosa were found to cause condensation and contraction of Allium cepa chromosomes, in addition to retardation and/or inhibition of mitoses and changed mitotic phases [31]. The difference of our study with that of Kuras *et al.* [31] is use of alkaloid fraction in the latter. Phytopreparations with immunostimulatory properties are known to increase the frequency of sister chromatid exchanges in cultured peripheral blood mononuclear cells, an effect associated with a slight increase of the [3H] thymidine uptake in the DNA of isolated lymphocytes. The induction of SCE is reported to be associated with the increase of cytokines interleukin (IL)-2 and tumor necrosis factor (TNF)-alpha [10]. These results demonstrate that Cat's clawappears to be mutagenic activity in Allium cepa and cultured peripheral blood mononuclear cells. While in our vivo experiments, there is no mutagenic effect. The results of nucleic acids observed in the present experiment also showed no effect of Cat's claw. However, there was discordance observed in our results on sperm abnormality and nucleic acid. There are no parallel studies available in the literature for a possible comparison; however, it is possible that the expression of any biochemical change will not be simultaneously observed at the cellular level.The effect on the decrease in the concentrations MDA after prolonged treatment showed the antioxidant potential of Cat's claw. Dreifus *et al.* [32] also reported that Uncaria tomentosa modify the oxidative stress and possess antioxidant properties. Nevertheless, Cat's claw lacked any effect on the testicular levels of NP-SH. These changes are attributed to the influence of alkaloid and steroid oxidants present in Cat's claw [1, 15]. The impact on MDA levels and lack of any effect on the NP-SH levels clearly reveal a balance between the oxidant and antioxidant potentials of Cat's claw, which has surfaced in the testicular cells. Nevertheless, our results on the oxidant potentials of Cat's claw testicular is not in harmony with the reported free radical scavenging activity of Cat's claw [5]. These authors reported Cat's claw to be an effective scavenger of free radical (1, 1-dipenyl-2-picrilhydrazil) in murine macrophages. However, we are aware that a better characterization of this antioxidant capacity requires model systems and methodologies involving the generation of diverse reactive species, namely superoxide anion, peroxyl and hydroxyl radicals, hydrogen peroxide and hypochlorous acid, as well as the evaluation of the potential ability to decrease them Halliwell [33].

Our study on the pituitary gonadal hormones significantly increased the human chorionic gonadotropin and decreased the testosterone levels in the plasma. A trend increase was also found in the levels of follicle stimulating hormone and estradiol. There are no parallel studies available on the male gonadal hormones. However, these changes are attributed to the depletion of MDA observed in the testis. Our results are supported by the data on reproductive toxicity in male mice. There are no parallel studies on endocrinology, however, these changes are attributed to the stimulation of immune system under the influence of alkaloids and steroids present in Cat's claw [1, 15]. Taken together, the treatment with Cat's claw has an impact on reduced fertility in female mice mated to treated male mice, it induces pre-implantation loss, causes changes in the sperm abnormality, reduced the MDA levels of the testis, increased the levels of human-chorionic gonadotropin and decreased the levels of testosterone. However there were no changes in testicular chromosomes and dominant lethal mutations. Further studies on biochemical changes and reproductive toxicity in other rodents are recommended before clinical experimentation.

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